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Rapid Bacterial Detection During Endodontic Treatment

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Abstract

Bacteria present in the root canal (RC) space following a root canal treatment (RCT) can lead to persistent infections, resulting in treatment failure and the need for re-intervention or extraction. Currently, there are no standardised methods in use to clinically detect bacterial presence within RC spaces. The use of paper point sampling and fluorescence staining was shown to be a rapid method, able to detect residual bacteria following treatment.

The study demonstrated that Calcein AM (CAM) proved to be a suitable dye for detecting vital bacteria within mature endodontic biofilms, with an improved sensitivity over CFU counting in a stressed biofilm model. Furthermore, in a clinical trial using primary RCTs,

53 infected teeth were sampled in-vivo and increased detection of vital cells was found when compared to CFU counting, highlighting the sensitivity of the technique in detecting low cell numbers.

Combining fluorescent staining and micro-spectroscopy with software-based spectral analysis, successful detection of vital cells from RCs was possible after 5 minutes of CAM incubation. Application of this technology during RCT has the potential to reduce persistent infections through vital cell detection and additional treatment. Furthermore, this technique could be applied to anti-microbial research and disinfection control in clinical settings.

Clinical trial registration number: 05/Q0705/051 (UK), approved by the South London Research and Ethics Committee.

Introduction

Root canal treatments (RCT) are undertaken to eradicate bacterial infections from root canals (RC), while retaining as much of the natural tooth as possible. One million RCTs are conducted under the British National Health Service each year, annually costing the General Dental Service £50.5 million (Lumley et al. 2008). Approximately 24% require secondary RCTs, due to persistence of microorganisms in the RC space (Siqueira 2001; Chávez de Paz 2007; Lumley et al. 2008; Anderson et al. 2012). During RCT, the debridement and removal of bacteria, as well as the shaping of the RC, takes place (see Appendix for additional information). The main difficulties in RC preparations lie in the

varying, unpredictable RC anatomy, which is often underestimated by conventional 2D radiographs (Peters 2004; Patel 2009). The resilient nature of bacterial biofilms, combined with often complex RC morphologies, make disinfection challenging, leading to a considerable number of persistent infections (Lumley et al. 2008), which are one of the main causes of RCT failure (Chávez de Paz 2007).

Current clinical practises aiming at detecting bacteria in the RC space rely on subjective observations such as clean, white dentine on the root canal files. Reproducible detection and quantification of bacteria, using methods such as colony forming unit (CFU) counting and polymerase chain reaction (PCR) (Kato et al. 2007; Sathorn et al. 2007; Anderson et al. 2012) are time consuming, making them unfeasible for routine intra-operative application. Another approach is to measure the auto-fluorescence from within the RC to differentiate infected from sound dentine. However, this relatively fast method relies on the fluorescence emission of biofilm by-products such as porphyrins and therefore lacks the ability to indicate the presence of vital cells and in particular, bacteria. Furthermore, it remains unclear whether the sensitivity of such auto-fluorescence measurements would be sufficient to detect very low quantities of bacteria (Giana et al. 2003; Sainsbury et al. 2009; Ho et al. 2010). Sato *et al.* propose application of a system using live/dead staining and a membrane filter for bacterial counting (Sato et al. 2012). Unfortunately, the procedure, despite being comparatively rapid, takes 30 minutes and requires additional collection and preparation of dentine samples, making it unfeasible for routine use in a clinical setting. A recent approach by Tan *et al*. utilises the detection of vital bacteria using adenosine triphosphate (ATP) activity. Although the ATP assay is rapid (~5 min) and

sensitive, the technique described requires additional sampling and root canal preparation before application of the ATP assay, increasing the overall time for treatment (Tan et al. 2015).

We have developed a new methodology based on the use of fluorescent dyes for the detection of vital bacteria, sampled using conventional endodontic paper points during RCTs. Both fluorescence spectroscopy and microscopy are commonly used methods in biological and chemical sciences and are established tools in bacterial biofilm analysis and detection (Moter and Göbel 2000; Takenaka et al. 2008; Wakamatsu et al. 2013). In “live/dead staining”, the chosen dyes will contrast vital from dead cells by using two fluorescent stains with different emission wavelengths. Ordinarily, they will exploit metabolic activity or compromised cell membranes and have a high affinity to cell structures such as nucleic acids (Tawakoli et al. 2012). By using non-specific vital cell staining, live cells in a range of bacteria involved in multi species RCTs can be detected (Shen et al. 2010; Shen et al. 2011; Tawakoli et al. 2012; Wakamatsu et al. 2013).

In this study, we first used confocal microscopy to compare the suitability of a number of fluorescent dyes for rapid detection of vital cells in a model endodontic *in-vitro* biofilm. As shown by Niazi *et al*. (Niazi, Clark, et al. 2014; Niazi, Al-Ali, et al. 2014), this biofilm consists of strains of selected pathogens, confirmed using 16S rRNA gene sequencing and quantitative viable counts.

To demonstrate the possibility of bench-side detection, we coupled a spectrometer to a fluorescence microscope with a custom filter cube. Further, using spectral un-mixing, emissions from the fluorescent dyes were shown to be distinguishable from substrate

auto-fluorescence, even at low fluorescence intensities. To demonstrate its clinical suitability, our methodology was applied to the detection of vital cells in a stressed *in-vitro* grown biofilm as a controlled model system, as well as to *ex-vivo* bacterial biofilms sampled with endodontic paper points during and post RC patient treatments (Figure 1). The use of established endodontic paper points minimises additional clinical steps during RCTs and therefore, does not impact the clinical treatment time.

[Insert Figure 1]

Figure 1: Outline of clinical detection: A) Sampling of the RC space, B) vital fluorescence staining of sample and C) rapid detection of fluorescent signal. D) Example of stained paper point with indicated detection area for spectral analysis.

The presented technique could be utilised to determine the endpoint of the endodontic chemo-mechanical debridement by assessing the removal of the endodontic pathogens from the main RC lumen in real time. Routine use could lead to a significant decrease in the need for re-treatments resulting in long term monetary savings to the dental services and enhanced confidence in a positive outcome of the RCT.

Methods

Establishment of a mature endodontic *in-vitro* biofilm

Replication of a multispecies biofilm consisting of typical RC pathogens required *in-vitro* culturing of a model biofilm system, as described by *Niazi et al.* (Niazi, Clark, et al. 2014;

Niazi, Al-Ali, et al. 2014). The strains used for the formation of the biofilm were *Propionibacterium acnes, Staphylococcus epidermidis, Actinomyces radicidentis* and *Streptococcus mitis,* which have been shown to be the predominant taxa from refractory endodontic lesions (Niazi et al. 2010). In addition, *Enterococcus faecalis* OMGS 3202, known be involved in RCT failures, was added to the biofilm (Dahlen et al. 2000). Bacterial isolates were revived from storage at - 80˚ C and cultured at 37˚ C in a MACS-MG-1000-anaerobic workstation (80% N, 10% H, 10% C02) before being transferred to modified fluid universal medium (mFUM) for anaerobic growth on autoclaved hydroxyapatite (HA) discs (Clarkson Chromatography Products Inc. USA). See Appendix and Appendix Table 1 for a detailed growth protocol and confirmation of present bacterial strains.

Characterisation and sensitivity of fluorescent stains to vital biofilms

The vast selection of fluorescent stains was narrowed to number of stains with various binding and fluorescence characteristics: Syto 9 (L10316 Filmtracer Live/Dead, Life Technologies, UK), Propidium iodide (PI), Filmtracer Red-Orange (F10319, Life Technologies, UK) and Calcein AM (CAM) (sc-203865, Santa Cruz Biotechnology, inc. USA, Texas). Details on the staining protocol and working solutions are given in the Appendix and Appendix Table 2. In addition to staining the vital biofilms, background auto-fluorescence measurements, as well as controls of stained HA discs, paper points (ProTaper universal paper points, Densply, UK) and non-vital biofilms were taken. Images were analysed by measuring the fluorescence intensity (Figure 2) using Image J 1.47v (Schneider et al. 2012), as described in Appendix Figure 1.

Simultaneous Image localisation and spectral detection

Image localisation and spectral analysis were carried out as described in the Appendix and Appendix Figure 2. In brief, a wide-field fluorescence microscope (Zeiss Axiovert 2000, Zeiss, Germany) was coupled to a fluorescence spectrometer (QE 65000, Ocean Optics, The Netherlands), allowing for image acquisition and simultaneous spectral recording. Spectral unmixing software then enabled to calculate the proportion of the calcein signal P_C, produced by vital cells. Spectral analysis was achieved by using pre-recorded base spectra as shown in Figure 3.

Detection of vital cells in stressed biofilms *in-vitro*

To evaluate the detection sensitivity in comparison to gold-standard CFU counting, biofilms grown on HA discs were exposed to 1% sodium hypochlorite (NaOCl) for 10 increasing durations (n=5), before being thoroughly rinsed with phosphate buffered saline. Following fluorescent staining, spectral analysis was carried out at 20 locations per disc. In addition, culturing and CFU counting was carried out for comparison with the fluorescence detection. A detailed protocol is outlined in the Appendix.

Detection of *ex-vivo* vital cells from patient root canals

Following ethical approval (05/Q0705/051), patients (n=53) undergoing primary RC treatments were selected and informed consent was taken. The teeth included in the study had been referred to a specialist centre for the treatment of endodontic infections, they all responded negatively to vitality tests and, with few exceptions, all presented pre-

operative apical radiolucencies. Endodontic paper points were used to sample the RC space at three time points during treatments: immediately after accessing the RC space, at the midpoint of the treatments (after instrumenting with shaping file (S2, ProTaper universal, Densply, UK) and pre-obturation. Spectral un-mixing software was used to analyse the fluorescently stained paper point samples (20 spectral readings at 500 µm intervals, starting from the tip). The proportional calcein signal ($\boldsymbol{P}_C$) was calculated and, using controls, a threshold of 4.35% was chosen as positive for bacterial detection (Figure 4). Furthermore, CFU counts were then conducted on all the samples after 7 days and compared with the proportional calcein signal, as shown in Figure 5. Sampling and sample analysis details are outlined in the Appendix and base spectra are for spectral unmixing are shown in Appendix Figure 3.

Results

Characterisation and sensitivity of fluorescent stains to vital biofilms

Visualisation of the vital biofilm was possible with all of the tested stains and stain concentrations. To compare the ability of each stain and concentration to specifically detect vital biofilms, as opposed to non-vital biofilms, we measured the ratio ($\boldsymbol{R_v}$) between the fluorescence of vital and non-vital biofilms. Similarly, the ratio ($\boldsymbol{R_S}$) between the fluorescence of vital biofilm and biofilm-free substrate was used to compare their ability to not stain the substrate.

Overall, CAM showed the highest ratios at all concentrations for all dyes (Figure 2A). The maximum ratio R_V = 11.7 was observed at a concentration of 15 µg/ml (Figure 2B). Above this concentration the, vital cell fluorescence intensity remained unchanged. Only minimal changes were observed for Syto 9 and Filmtracer Red-Orange at any of the concentrations (Figure 2A), where the maximum signal of 1.8 and 1.3 were measured at 50 and 10 µg/ml respectively (Figure 2B). In the biofilm paper point ratio R_S the same high signal was observed for CAM at 15 µg/ml (Figure 2C,D). Fluorescence intensity of each stain at all the incubation times and on all the substrates is shown in Appendix Figures 4-6.

An ANOVA Holm-Sidak statistics test showed that CAM staining produced a significantly higher ($p<0.001$) vital/non-vital biofilm staining ratio when compared to both Styo 9 and Filmtracer Red-Orange (Figure 2A&B). Staining vital biofilms compared to the paper point substrate was also shown to be significantly higher than Syto 9 and Filmtracer Red-Orange ($p<0.001$) (Figure 2D). These experiments have established the most suitable stain and optimal concentrations for all future experiments outlined in this paper as CAM at 15 µg/ml.

[Insert Figure 2]

Figure 2: Characterisation of fluorescent stains. A) Comparison of fluorescence ratio of stained vital biofilms to stained dead fixed biofilms at different concentrations. B) The ratio of stained vital biofilms to stained dead fixed biofilms at each stains optimal concentration. C) Comparison of fluorescence ratio of stained vital biofilms to stained sterile paper points. D) The ratio of stained vital biofilms to stained sterile paper points at the chosen

concentrations. Statistics were performed using a one-way ANOVA test with the Holm-Sidak method n=9, error bars represent standard deviation, * = p< 0.001).

Detection of vital cells in stressed biofilms *in-vitro*

The optical biofilm sampling and detection method was compared to the gold standard of CFU counting by using nutritionally stressed biofilms, which were exposed to NaOCl for increasing durations (Figure 3). An immediate 3.3-fold drop in proportional calcein signal was observed after 2 sec of NaOCl exposure (Full scale in Appendix Figure 7). As expected, increased exposure durations resulted in further decrease of the proportional calcein signal. However, detection of this signal remained possible for up to 40 sec of exposure with NaOCl (Figure 3C). After being exposed for 200 sec, it dropped below to the non-vital positive control, indicating a complete loss in viability (Appendix Figure 7).

In comparison to the calcein signal, the CFU counts showed a far stronger drop (861 fold) after 2 sec of NaOCl exposure and the stressed biofilms completely lost the ability to form colonies after 15 sec of NaOCl exposure. These results indicate that the bacteria within the biofilm are losing the ability to form CFUs but remain in a detectable vital state for up to 40 sec exposure, as observed in other studies (Shen et al. 2010; Shen et al. 2011).

These measurements were repeated by sampling the same biofilms with endodontic paper points and interestingly, detection of residual vital cells was improved. These results suggest that condensing the biomass into a small surface area maximises the

concentration of stained cells and increases the sensitivity of calcein detection. This advantage would be replicated during clinical sampling of the RC (Appendix Figure 8).

[Insert Figure 3]

Figure 3: Spectral analysis. A) Normalised base spectra used for spectral un-mixing (green = fluorescence in presence of vital cells (positive signal), red = substrate and non-vital biofilm. B) Example of experimental spectra with fitted data. C) Detection of vital bacteria using the proportion of positive signal from spectral un-mixing.

Detection of *ex-vivo* vital cells from patient root canals

Samples were taken from 53 patients during RCTs with endodontic paper points and tested for vital bacteria using the described methodology. Sterile paper points identified a broad autofluorescence spectrum, which increased in intensity as the taper of the paper point increased (Appendix Figure 9).

In-vivo detection of vital bacteria inside RCs during RCTs was carried out by sampling immediately after access, post shaping and pre-obturation. Figure 4 shows an example of a stained sample from a clinical case with a very high proportional calcein signal at the tip, which decreases along the paper point. The strong signal at the tip (top insert) can be attributed to the tip of the paper point accumulating bacteria on entry to the apex, as well as a larger amount of bacteria in the apex of the tooth. However, further along the paper point shaft, the contribution of the background autofluorescence is dominant (bottom insert).

[Insert Figure 4]

Figure 4: Signal detection from a patient sample. The spectral un-mixing is shown at two different points on the patient sample. The low signal from the sterile stained paper points and the determined detection threshold for sampling are indicated.

Typically, a large amount of signal was detected in the primary sample (post access) with decreasing signal in the secondary sample (post shaping) and in the final sample (pre-obturation) to the same level observed by the negative control. However, in some cases the proportional calcein signal was greater than the 4.35% detection threshold, indicating the presence of remaining vital bacteria in the apical portion of the RC post RCT.

Paper points positive for vital cells always maintained maximum signal towards the tip (Appendix Figure 10). To compare the different RCT stages, the first three recorded locations along the paper point (starting at the tip) were averaged. The data demonstrated that roots with detection over the threshold decreased in number with the progression of the treatment: from 74.6% post access, to 29.8% after shaping and 18.4% pre-obturation (Figure 5). The decrease in a detectable vital signal shows that during the chemo mechanical treatment, the majority of bacteria are removed. The proportional calcein signal distribution of the pre-obturation samples are shown in Appendix Table 3, indicating the majority of roots being below detection limits. Despite the lower numbers of RCs with detectable signal in the apex at the end of the treatment, clinically this results in 35.8% of teeth sampled showing a signal above detection threshold (Appendix Figure 11). Furthermore, fluorescence detection proved to be more sensitive than CFUs in the

detection of vital cells; A calcein signal was detected pre obturation in 18.4% of roots compared to 6.1% using culturing (Figure 5).

[Insert Figure 5]

Figure 5: Clinical trial consisting of 53 RCTs: Percentage of roots in which a signal was detected at different time points throughout the treatment, as well as comparison to CFU detection, n = 114.

Discussion

Persistent microbial infections and bacteria remaining in the RC space following treatment are one of the main causes of failure of RCTs (Siqueira 2001; Chávez de Paz 2007). This study aimed to develop an optical, fluorescence-based chair-side method, which allows clinicians to objectively and rapidly detect residual bacterial contamination in the RC space. *In-vivo* sampling with endodontic paper points combined with *ex-situ* staining and analysis results in a rapid method which causes no disruption to the clinical workflow, inconvenience or discomfort to the patient.

Unspecific vital cell stains enable the detection of a wide range of bacteria involved in multispecies infections of the RC space (Costerton et al. 1999; Davey et al. 2000; Hall-Stoodley et al. 2004; Shen et al. 2010). Characterisation of various fluorescent dyes showed CAM to be the most suited in detecting vital bacteria in a mature, nutritionally stressed multispecies biofilm after just 5 minutes of incubation with minimal background staining. Low fluorescence ratios for R_s and R_v produced by the others stains can be

attributed to staining of the absorbent paper point and the 5 minutes incubation being insufficient reaction time (Figure 2). The biofilm used consisted of the predominant bacteria recovered from RCs of teeth affected by apical periodontitis (Niazi et al. 2010) including *E.Faecalis* (Lima et al. 2001; Stuart et al. 2006).

We have developed a setup which combines fluorescence spectroscopy and microscopy for simultaneous localisation and spectral analysis of vital bacteria using CAM. Applying spectral un-mixing enables accurate quantification of both the stained biofilm and the substrate (endodontic paper point or HA disc) auto-fluorescence. A proof of principle study, subjecting biofilms to 1% NaOCl with increasing durations, showed our method to be more sensitive to very low quantities of vital cells, compared to CFU counting. Bacterial biofilms have numerous mechanisms of reacting to environmental stress, including down regulating metabolism and entering a vital but non-culturable (VBNC) (Mah and O'Toole 2001; Stewart and Costerton 2001; Shen et al. 2011), not detectable by CFU counting. However, as shown in Figure 3C, the bacterial metabolism is sufficient to hydrolyse CAM into the fluorescent calcein, enabling fluorescent detection even when the cells have lost the ability to form CFUs. This is of great importance when aiming to detect bacteria deep within the nutrient poor RC space where surviving biofilms may enter a VBNC state whilst still causing inflammation (Shen et al. 2010).

Our clinical trial, consisting of 53 sampled RCTs, demonstrated the feasibility of this technique in a practical clinical setting. RCs were sampled and spectrally analysed immediately after accessing the RC space, after shaping and pre-obturation. Spectral analysis of control stained sterile paper points lead to a threshold of 4.35 (Figure 4). A

clear decrease in vital biofilm detection from the samples taken immediately after access compared to samples taken during and post-treatment was observed (Figure 5). While the majority of pre-obturation samples did not exceed the detection threshold, remaining vital bacteria were detected at the apex of the RC space in 18.4% of roots. Furthermore, similarly to the *in-vitro* detection of bacteria in a VBNC state, the clinical trial showed that fluorescent analysis led to bacterial detection in more cases than CFU counting, which only identified residual bacteria in 13.2% of teeth. Interestingly, the detection of vital cells in 19 out of 53 of sampled teeth (35.8%) exceeds the percentage of RCT failures generally suggested by the literature, which are in the region of 20 to 25 % when assessed using periapical radiographs (Ng et al. 2007; Lumley et al. 2008). However, there is substantial agreement between the percentage of positively sampled teeth in the present study and rate of periapical pathosis observed in CBCT studies in primary RCTs, where detection at the 1-year recall was 37.5% (Patel et al. 2012). The correlation between fluorescence detection and treatment failure is currently being examined in a further clinical trial to demonstrate the relevance of residual bacteria. Sampled patients will be recalled one-year post obturation for CBCT evaluation.

Since paper point sampling is restricted to the pathway created by of the endodontic instruments, detection within inaccessible structures such as lateral canals may not be possible. However, endodontic paper points have the benefit of being highly absorbent and flexible, enhancing the detection of vital cells in the RC apex, entrance to lateral canals and walls which remain untouched by mechanical instrumentation (Peters et al. 2003).

Our study demonstrates the potential advantages and clinical relevance of fluorescent staining in combination with spectral analysis. Compared to more conventional microbiology techniques such as CFU counting or PCR (Kato et al. 2007; Sathorn et al. 2007; Anderson et al. 2012), the approach significantly reduces the processing time required, making it a viable technique for introduction into the current workstream of dental practices, capitalising on otherwise discarded biological material. As opposed to recently published techniques (Sato et al. 2012; Tan et al. 2015), we demonstrate the detection of the residual bacteria directly on paper points, avoiding additional preparation of the sample or the root. Valuable time is saved as measurements can be achieved in 5 minutes, making the technique clinically relevant for direct chair side detection.

Our unbiased, rapid and quantitative methodology was first proven on stressed *in-vitro* biofilms. Furthermore, it was successfully applied to detecting *ex-vivo* vital bacteria in samples taken during RCTs. The potential to minimise persistent infections, would reduce treatment costs and avoid an unnecessary secondary visit when no bacteria are detected. This technology also has the potential to be applied to other areas, both clinically and in research. Research applications could include the evaluation of biofilm disinfection methods in areas of peri-implantitis and bone infection as well as general antibiotic and antimicrobial research. Clinically, the future development and application of more specific vital fluorescent stains could be applied to *ex-vivo* detection from patients in a wide range of biological infections, ranging from wound or respiratory to implant related infections and contaminations.

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We confirm that this work is original, not under publication consideration elsewhere and is free of conflict of interest.

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